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Subunit Composition and O_2 Binding of the Crustacean Hemocyanins: Interspecific Relationships

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Abstract. The monomeric subunit composition and oxygen binding properties of hemocyanins were examined in 9 taxonomic groups of 43 crustacean species and 1 hybrid. In native polyacrylamide electrophoresis the banding pattern was highly species specific, even in closely related congeners. In less closely related taxa, there was little apparent relationship between phylogenetic affinity and banding pattern.

Within a taxonomic group, pH dependence was the most highly conserved and O_2 affinity was the most diverse of the O_2 binding properties investigated. In congeneric but not sibling species, O_2 affinity was more highly correlated with an environmental variable such as temperature than with phylogenetic affinity. Only in very closely related groups found in similar environments were different O_2 binding properties correlated with differences in particular electrophoretic bands.

Introduction

Crustacean hemocyanins (Hcs) exist *in vivo* as hexamers (1×6 monomers) and multiples of hexamers, most often a pair (2×6-mers). Although the polypeptide composition of both artificial and natural 1×6-mers can be homogeneous, the native blood in a species contains more than one, and up to a dozen or more different, monomeric chains (reviewed by Markl, 1986, and Markl and Decker, 1992; see also Callicott and Mangum, 1993; Mangum and Greaves, unpub. data). This condition, which is known as monomeric subunit heterogeneity, is a necessary prerequisite to differences in polypeptide chain composition between individuals of a species, which is known as monomeric subunit polymorphism.

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Using electrophoretic heterogeneity and immunological relatedness in combination, Markl (1986) described distinct patterns of monomeric subunit composition in different arthropod families, both crustacean and arachnid. He formulated three discrete immunological categories of chains, designated alphas, betas, and gammas. When separated by native polyacrylamide gel electrophoresis (PAGE), members of each category exhibit migration rates that are characteristic of their category.

Subunit composition can influence intrinsic O_2-binding properties. Artificial components such as isolated monomers (Jeffrey and Treacy, 1980) or homohexamers of them (Johnson *et al.*, 1987) differ in O_2 binding. More important in the present context, the respiratory properties of natural Hcs with different subunit compositions can differ within a polymorphic species or between sibling species. In addition, the differences can be attributed to particular chains and, in one case, their effects on oligomerization (Mangum and Rainer, 1988; Mangum *et al.*, 1991; Mangum, 1993a, b, 1994). On the other hand, not all different Hc morphs, either within or between species, have appreciably different functional properties (Mangum, 1993a, b).

Markl's (1986) investigations were designed to elucidate broad phylogenetic relationships of Hcs representing taxonomic categories ranging from families to subphyla. Because his sample was composed of, at most, a few species per family, the patterns he described may or may not be characteristic of the families. Few interspecific comparisons of PAGE banding patterns, much less respiratory properties, have been made under common experimental conditions. In addition, the level of evolutionary divergence at which subunit composition and respiratory properties begin to differ is not known.

Here we present PAGE and O_2-binding data for nine taxonomic sets of crustaceans, primarily decapods, of various degrees of relatedness. The taxonomic sets differ

in their composition. The separation ranges from a natural hybrid and its sibling parental species, to sibling species, to nonsibling and also sympatric congeners, to different genera, to closely related families.

Materials and Methods

The species investigated are listed in Table I. Most representatives were collected and bled by the present authors. *Pagurus impressus, P. lacustris,* and *Uca virens* were purchased from a commercial supplier and then bled by us. The Pacific coast species of *Cancer,* the two species of *Chaceon* and *Menippe,* and the *Menippe* hybrid were bled by colleagues and the fresh material was shipped to us within 24 h. *Callinectes arcuatus, C. toxotes, Portunus spinnemanus, Panopeus lacustrus, Cataleptodius floridanus, Uca inversa,* and the two species of *Gnathophausia* were bled by colleagues and the material was shipped to us frozen.

Blood samples

Blood was taken from the infrabranchial sinuses into a hypodermic syringe and allowed to clot in a tissue grinder. Blood from all available individuals of a species (see Table III for the actual numbers) was pooled. The pool was then homogenized and centrifuged, and the clot was discarded. A small aliquot of serum was frozen for PAGE, and O_2 binding of the remainder was determined immediately.

Most of the O_2-binding measurements were performed within 1–2 days of bleeding, on material that had not been frozen. Because freezing can lower cooperativity (Morris, 1988), we have chosen not to report cooperativity values for most of the Hcs that had been frozen. We report values for *Callinectes arcuatus* and *C. toxotes,* however, because an investigation of *Callinectes sapidus* Hc revealed no effect of similarly brief periods of freezing (Mangum *et al.,* 1991).

Electrophoresis

The frozen material was thawed, and its Hc concentration was estimated by absorbance of dissociated subunits at the active site (338 nm). It was then diluted to about 5 mg l^{-1} with a buffer (0.05 mol l^{-1} Tris + 0.05 mol l^{-1} EDTA, pH 8.9) that dissociates native oligomers into their constituent monomers. The Hc monomers were then separated by native PAGE; the gels, reagents, and other conditions were those detailed by Hames and Rickwood (1985). This is the most sensitive procedure available for separating the monomers of these proteins; for comparison with alternative methods, please see Markl *et al.* (1979), Brenowitz *et al.* (1981), and Stöcker *et al.* (1988).

In most cases, the best separation was obtained on 12.5% gels. Higher and lower gel concentrations were also employed when we encountered dense bands that suggested more than one chain with similar migration rates. Three or more amounts of Hc were first examined to determine the optimal level for each species; the level chosen was similar within a taxonomic set, but it differed between sets. The Hcs from each set were subsequently analyzed on the same gel.

Most gels were stained with Coomassie Blue alone. After the banding pattern for a species was ascertained, a gel was prepared for the detection of Cu in the bands. The presence of Cu was examined by noting the quenching of fluorescence generated by bathocuproine sulfonate (Bruyninckx *et al.,* 1978). In the present investigation, EDTA was omitted from the dissociating buffer (N.B. Terwilliger, pers. comm.). Due to the lower sensitivity of the bathocuproine sulfonate procedure, the Hc concentration was raised by a factor of 6. The positions of the Cu-positive bands were marked and the gels were then stained with Coomassie Blue to verify their correspondence to those previously noted with Coomassie Blue alone.

The number of bands in a species was ascertained by visual inspection of the gels on a light table. In most species, the relative amounts of material in each band were estimated from densitometric scans. A few species were investigated in a laboratory in which no densitometer was available. Gels were initially scanned with a Gelman integrating densitometer. Although the width of the scan produced by this instrument is not adjustable, it gave the best resolution of the individual peaks. It expresses the area of each peak as an integram (a number of spikes that varies directly with peak area), which permits calculation of the percentage of the total material. These calculations (in Table II) include only the Cu-positive material. To aid in the examination of gel photographs, additional scans were made with a Shimadzu densitometer. The widths of these scans were precisely compressed so that the peaks were aligned with their corresponding bands on the gel. Photographs of the gels and their corresponding scans can be found in Reese (1989).

O_2 binding

Hcs were stripped of organic modulators by 12 h dialysis with Spectrapor high-speed membranes, against a physiological saline. Where possible, the saline was based on the ionic composition of a blood representative of a taxonomic set. If no information on ionic composition was available, seawater was used. The dialyzed Hcs were then diluted to the optimal absorbance range with 0.05 mol l^{-1} Tris-buffered saline, and the pH was adjusted with HCl and NaOH.

Table I

The classification, geographic distribution and habitats of the species

Species	Habitat	Geographic Range
Order Decapoda		
Infraorder Anomura		
Family Diogenidae		
Clibinarius vittatus (Bosc)	subtidal, 0 to 22 m	Virginia to Brazil
Family Paguridae		
Pagurus inpressus (Benedict)	subtidal, 1 to 33 m	North Carolina to Florida
Pagurus longicarpus Say	intertidal to 200 m	Massachusetts to Florida
Pagurus pollicaris (Say)	subtidal to 200 m	New Brunswick to Florida
Infraorder Brachyura		
Family Cancridae		
Cancer antennarius Stimpson	0 to 20 m	British Columbia to Baja California
Cancer anthonyii Rathbun	0 to 20 m	Monterey Bay to Gulf of Baja California
Cancer borealis Stimpson	intertidal to 800 m	Nova Scotia to Florida
Cancer irroratus Say	subtidal, 0 to 575 m	Labrador to Florida
Cancer magister (Dana)	shallow subtidal	Aleutians to Monterey Bay
Cancer productus (Randall)	shallow subtidal	Kodiak to Baja California
Family Geryonidae		
Chaceon fenneri (Manning & Holthius)	300 m +	Gulf of Mexico
Chaceon quinquedens (Smith)	300 to 700 m +	Gulf of Mexico
Family Grapsidae		
Sesarma cinereum (Bosc)	semiterrestrial	Maryland to Mexico
Sesarma reticulatum (Say)	semiterrestrial	Massachusetts to Florida
Family Majidae		
Libinia dubia H. Milne-Edwards	subtidal, 0 to 46 m	Massachusetts to Cuba
Libinia emarginata (Leach)	subtidal, 0 to 49 m	Novia Scotia to Gulf of Mexico
Family Ocypodidae		
Ocypode quadrata (Fabricius)	semiterrestrial	Rhode Island to Brazil
Uca crenulata coloradensis (Lockington)	semiterrestrial	Gulf of Baja California
Uca inversa Hoffman	semiterrestrial	Indian Ocean and Arabian Sea from Natal to Pakistan
Uca musica musica Rathbun	semiterrestrial	Baja California to San Blas
Uca princeps monilifera Crane	semiterrestrial	Gulf of Baja California
Uca pugilator (Bosc)	semiterrestrial	Massachusetts to Florida
Uca pugnax (Smith)	semiterrestrial	Massachusetts to Florida
Uca minax (Le Conte)	semiterrestrial	Massachusetts to Florida
Uca virens (Salmon & Atsaides)	semiterrestrial	Florida to Mexico
Family Portunidae		
Ovalipes ocellatus (Herbst)	subtidal, 0 to 95 m	Northumberland Straits to Georgia
Portunus gibbesii (Stimpson)	subtidal, 0 to 393 m	Ma. to Gulf of Mexico; French Guiana
Portunus spinimanus (Latreille)	subtidal, 0 to 91 m	New Jersey to Brazil
Callinectes arcuatus Ordway	subtidal, 0 to 28 m	Southern California to Peru
Callinectes bellicosus Stimpson	subtidal, 0 to 18 m	San Diego to Matzatlan
Callinectes ornatus Ordway	subtidal, 0 to 75 m	Virginia to Brazil
Callinectes sapidus Rathbun	subtidal, 0 to 90 m	Nova Scotia to Argentina
Callinectes similis Williams	subtidal, 0 to 392 m	Delaware Bay to Colombia
Callinectes toxotes Ordway	subtidal, 0 to 27 m	Baja California to Peru
Family Xanthidae		
Menippe adina Williams & Felder	shallow subtidal	Northern and western Gulf of Mexico
Menippe mercenaria (Say)	subtidal, 0 to 54 m	North Carolina to Mexico; Jamaica
Hybrid		Northwestern Florida
Panopeus herbstii H. Milne-Edwards	intertidal, to 22 m	Massachusetts to Brazil
Panopeus obesus (Smith)	intertidal	North Carolina to Florida
Panopeus lacustris Desbonne	intertidal to shallow subtidal	Bermuda, southern Florida to Brazil
Eurypanopeus depressus (Smith)	intertidal to 48 m	Massachusetts to tropics
Cataleptodius floridanus (Gibbes)	intertidal	Bermuda, southern Florida to Brazil
Order Mysidacea		
Gnathophausia gigas (Willemoes-suhm)	mesopelagic	North Pacific Ocean
Gnathophausia ingens (Dorhn)	mesopelagic	North Pacific Ocean

O_2 binding of all but a few Hcs was determined tonometrically at atmospheric pressure (Burnett, 1979), with precision mixed, humidified gases passed through tonometers in a rapidly shaken water bath (± <0.5°C); absorbance was determined with a Milton Roy Spectronic 501 spectrophotometer. The remaining Hcs, designated as such in Figure 2 and Table III, were examined with the cell respiration procedure (Mangum and Lykkeboe, 1979). In both cases P_{50} and n_{50} were estimated from Hill plots, using linear regions of the data in the region of 50% oxygenation. Bohr plots (log P_{50} *vs.* pH) were described by regression lines, and 95% confidence intervals were constructed around the lines and their slopes. Many of the data sets differ significantly throughout the pH range examined; a smaller number are statistically indistinguishable throughout the range examined. These relationships are clear from the Bohr plots. Many, however, differ significantly in only a portion of the pH range, which is indicated in the Results section. Cooperativity values were compared by using a Student's *t* test.

In vivo respiratory variables

Blood was taken anaerobically into a hypodermic syringe from the pericardial and infrabranchial sinuses, and pH and PO_2 were measured with a Radiometer BMS1 Blood Gas System. Because the samples were often small, the suction line was disconnected from the pH capillary electrode, and a syringe was used instead to position the material.

Results

Hc subunit composition

We have attempted to accurately represent the species investigated by choosing high-frequency phenotypes for those known to be polymorphic (Mangum, 1990, 1993a; Callicott and Mangum, 1993; Mangum and Greaves, unpub. data; C. P. Mangum and K. T. White, unpub. data). As discussed below, in no case known thus far can a morph of one species be confused with a morph in another. We note that one species (*Cancer productus*) is polymorphic (Wache *et al.*, 1988), but the polymorphism has not been characterized. In all likelihood, other members of our sample will also prove to be polymorphic.

Figures 1A and B summarize the banding patterns found in each species and allow direct comparison of the electrophoretic behavior of the monomers composing most of the Hcs in our sample. A few species were investigated after these gels were prepared; they are shown in Fig. 1C. All bands were assigned numbers in order from the top (anode) to the bottom (cathode) of the gel, with no implication that the same number in different species reflects co-migration. The relative amounts of the Cu-containing bands in each species are given in Table II.

1. *Anomuran hermit crabs: Diogenidae and Paguridae.* The Hc of the diogenid *Clibinarius vittatus* consistently exhibited two electrophoretic bands in apparently equal quantities. Visual inspection of the gels clearly revealed a light area in the middle and a corresponding constriction at the edges of the single dark band in Fig. 1A (lane 1); however, the separation does not show in this photograph. Regardless, the pattern differed unambiguously from that of the three pagurid Hcs, each of which had additional bands (Fig. 1, lanes 2–4). Most bands in the pagurids were more cathodic than those in *C. vittatus*. Each pagurid species clearly contained at least one band that did not co-migrate with a band found in the others. Although several of the bands in *C. vittatus, P. pollicarus,* and *P. longicarpus* did co-migrate, the proportions of the co-migrants differ considerably (Table II).

2. *Brachyurans: Cancridae.* The subunit compositions of the *Cancer* Hcs did not resemble one another very closely (Fig. 1B, lanes 1–6). Thus, it is unlikely that a morph of *C. productus* will prove to resemble that of any other species. *C. borealis* and *irroratus* Hcs each separated into three different bands, whereas *anthonyi* and *productus* Hcs separated into four, and *antennarius* and *magister* Hcs separated into five bands (see also Larsen *et al.*, 1981). Each Hc exhibited electrophoretically unique bands.

As in *Cancer pagurus* (Markl, 1986), the bands in the present sample of cancrids fell into three more or less distinct groups: an anodic group, an intermediate group, and a cathodic group. They may correspond to Markl's (1986) immunological categories, in the order beta, alpha, gamma. Within each group, some of the bands migrated at identical rates, and the migration rates of others differed only slightly. In all cases, however, the proportions of the co-migrants are considerably different (Table II).

3. *Grapsidae. Sesarma cinereum* Hc separated into two bands, whereas *S. reticulatum* Hc separated into three (Fig. 1A, lanes 13–14). Only band 2 in each species co-migrated.

4. *Majidae.* The Hcs of *Libinia dubia* and *emarginata* each exhibited four bands (Fig. 1B, lanes 9–10). In both species the most cathodic band predominated. Moreover, the position of this predominant band was quite different in each species, although some of the minor bands co-migrated.

5. *Ocypodidae. Ocypode quadrata* Hc (Fig. 1A, lane 19) contained four distinct bands (see also Markl, 1986; Johnson, 1987), none of which co-migrated with any bands found in the genus *Uca* (Figs. 1A, lanes 15–18, and Fig. 1C, lanes 5–7). The present material from *U. pugilator, U. pugnax,* and *U. virens* all contained four Cu-positive bands that migrated similarly but not exactly. The relative amounts of material are quite distinct in each species (Table II). Despite repeated attempts and with dif-

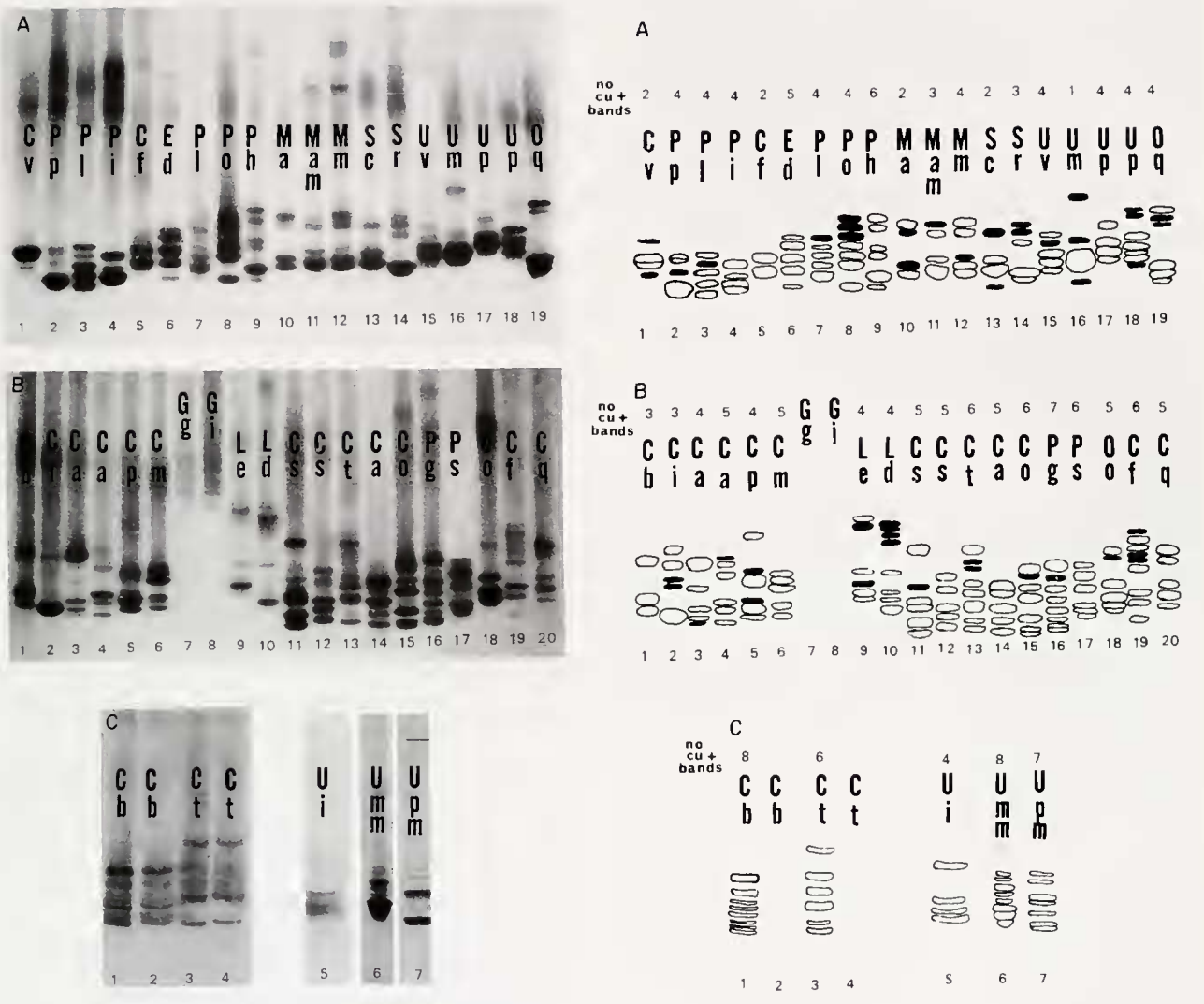


Figure 1. Native PAGE banding patterns of dissociated hemocyanins examined in the present investigation. Left: Photographs of gels summarizing the nine taxonomic clusters. Lane nos. at bottom. Right: Diagrammatic representations of photographs. Lane nos. at bottom. Number of Cu-positive (Cu +) bands at top. Cu-negative bands are blackened.

Panel A, from left to right beginning with lane 1: [1] *Clibinarius vittatus*, [2] *Pagurus pollicaris*, [3] *P. longicarpus*, [4] *P. impressus*, [5] *Cataleptodius floridanus*, [6] *Eurypanopeus depressus*, [7] *Panopeus lacustris*, [8] *P. obesus*, [9] *P. herbstii*, [10] *Menippe adina*, [11] *Menippe adina–mercenaria* hybrid, [12] *M. mercenaria*, [13] *Sesarma cinereum*, [14] *S. reticulatum*, [15] *Uca virens*, [16] *U. minax*, [17] *U. pugnax*, [18] *U. pugilator*, [19] *Ocypode quadrata*.

Panel B, left to right: [1] *Cancer borealis*, [2] *C. irroratus*, [3] *C. anthonyi*, [4] *C. antennarius*, [5] *C. productus*, [6] *C. magister*, [7] *Gnathophausia gigas*, [8] *G. ingens*, [9] *Libinia emarginata*, [10] *L. dubia*, [11] *Callinectes sapidus*, [12] *C. similis*, [13] *C. toxotes*, [14] *C. arcuatus*, [15] *C. ornatus*, [16] *Portunus gibbesii*, [17] *P. spinnemanus*, [18] *Ovalipes ocellatus*, [19] *Chaceon fenneri*, [20] *C. quinquedens*.

Panel C, left to right: *Callinectes bellicosus* (high conc.), *C. bellicosus* (low conc.), *C. toxotes* (high), *C. toxotes* (low), *Uca inversa*, *U. musica musica*, *U. princeps monilifera*.

ferent gel concentrations, we were unable to demonstrate more than one Cu-positive band in *U. minax* Hc, although several additional bands that were not Cu-positive clearly appeared on the gels.

Hcs from *U. musica musica*, *U. princeps monilifera*, and *U. inversa* were electrophoresed on the same gel with material from *U. pugilator* (Mangum, 1993a; Callicott and Mangum, 1993) to ascertain relative positions of the bands. *U. musica musica* Hc separated into eight bands, *U. princeps monilifera* Hc separated into seven bands, and *U. inversa* Hc separated into four bands (Fig. 1C, lanes 5–7). The major bands were diagnostic of each

Table II

Relative amounts (%) of electrophoretic bands

Species (abbr. #)*	Subunit Number 1	2	3	4	5	6	7
Infraorder Anomura							
Clibinarius vittatus (Cv, A1)	*ca.* 50†	*ca.* 50†					
Pagurus impressus (Pi, A4)	24	14	31	31			
Pagurus longicarpus (Pl, A3)	9	24	37	30			
Pagurus pollicaris (Pp, A2)	12	11	39	39			
Infraorder Brachyura							
Cancer antennarius (Ca, B4)	3	4	48	31	14		
Cancer anthonyi (Ca, B3)	64	4	17	15			
Cancer borealis (Cb, B1)	26	39	35				
Cancer irroratus (Ci, B2)	18	22	60				
Cancer magister (Cm, B6)	13	26	40	12	9		
Cancer productus (Cp, B5)	11	19	46	24			
Family Grapsidae							
Sesarma cinereum (Sc, A13)	40	60					
Sesarma reticulatum (Sr, A14)	18	41	41				
Family Majidae							
Libinia dubia (Ld, B10)	17	16	19	48			
Libinia emarginata (Le, B9)	11	23	20	27			
Family Ocypodidae							
Ocypode quadrata (Oq, A19)	20	42	16	22			
Uca pugilator (Up, B18)	25	25	18	32			
Uca pugnax (Up, B17)	26	31	36	7			
Uca minax (Um, B16)	100						
Uca virens (Uv, B15)	5	33	44	18			
Family Portunidae							
Ovalipes ocellatus (Oc, B18)	12	32	31	21	4		
Portunus gibbesii (Pg, B16)	11	11	7	10	11	23	27
Portunus spinimanus (Ps, B17)	9	22	14	14	26	15	
Callinectes arcuatus (Ca, B14)	33	15	28	13	11		
Callinectes ornatus (Co, B15)	13	8	21	25	19	14	
Callinectes sapidus (Cs, B11)	10	25	18	29	0	18	
Callinectes similis (Cs, B12)	16	6	32	23	23		
Callinectes toxotes (Ct, B13)	21	1	22	36	4	16	
Family Xanthidae							
Menippe adina (Ma, A10)	34	66					
Menippe mercenaria (Mm, A12)	16	16	38	30			
Hybrid (Mam, A11)	14	15	71				
Panopeus herbstii (Ph, A9)	14	19	11	13	31	1	
Panopeus obesus (Po, A8)	13	30	26	31			
Panopeus lacustris (Pl, A7)	13	34	25	28			
Eurypanopeus depressus (Ed, A6)	10	24	35	25	6		
Cataleptodius floridanus (Cf, A5)	50	50					
Family Geryonidae							
Chaceon fenneri (Cf, B19)	24	5	6	43	19	3	
Chaceon quinquedens (Cq, B20)	55	13	5	22	5		

* Species abbreviation and lane number used in Fig. 1.

† Estimated by eye.

species of *Uca*, although some of the minor bands co-migrated.

6. *Portunidae.* The subunit composition of *Callinectes sapidus* Hc is polymorphic (Mangum and Rainer, 1988; Mangum, 1990; Mangum *et al.*, 1991), as well as heterogeneous (Mason *et al.*, 1983; Johnson *et al.*, 1984; Stöcker *et al.*, 1988). The highly variable band 5 was not found in the material examined here; for comparison with Fig. 1B, lane 11, see scans in deFur *et al.* (1990). The degree of heterogeneity found earlier in *C. sapidus* appears to be characteristic of the portunid Hcs (Fig. 1B, lanes 11–15). The subunit patterns were distinct in each of the three genera examined. *Ovalipes ocellatus* Hc (Fig. 1B, lane 18) separated into five bands, one of which was anodic to any in *Portunus* (Fig. 1B, lanes 16–17). *Callinectes sapidus* (Fig. 1B, lane 11) and *C. toxotes* (Fig. 1B, lane 13 and 1C, lanes 3–4) Hcs had even more anodic (and also co-migratory) bands. A number of the bands in *P. gibbesii* co-migrated with those of *P. spinimanus* (Fig. 1B, lanes 16 and 17), although the relative amounts of these bands are quite different (Table II). Other bands were qualitatively distinct as well. Similarly, several bands among the *Callinectes* congeners co-migrated (Fig. 1B, lanes 11–15). *C. bellicosus* and *C. toxotes* are of particular interest (see Discussion); although four bands co-migrated, each Hc exhibited diagnostic bands, and the proportions of most co-migrants differ (Table II), which is also true of the other members of the genus. At least one band in *Portunus gibbesii* (Fig. 1B, lane 16) co-migrated with one in *Callinectes ornatus* (lane 15).

7. *Xanthidae.* The Hcs from the mud crabs were clearly distinctive (Fig. 1A, lanes 7–9). *P. herbstii* Hc separated into at least six bands, whereas *P. obesus* and *lacustris* Hcs each separated into four. Several bands co-migrated. As in the other families, however, the relative amounts of the co-migrants are not at all similar (Table II), and many bands were unique to a particular species.

Eurypanopeus depressus Hc (Fig. 1A, lane 6) contained at least five bands, four of which co-migrated with bands found in the three species of *Panopeus;* one band, however, was clearly unique to *E. depressus. Cataleptodius floridanus* (Fig. 1A, lane 5) Hc contained only two Cu-positive bands, both of which co-migrated with, and occur in concentrations similar to, bands in *E. depressus.* In *C. floridanus,* however, there was no sign of co-migrants with the remaining three *E. depressus* bands.

The sample of *Menippe adina* Hc contained only two bands that were clearly positive for Cu, whereas the Hc of its sibling *M. mercenaria* contained four (Fig. 1A, lanes 10–12). Both bands of *M. adina* Hc co-migrated with *M. mercenaria* bands. The relative amounts differ, though not dramatically. The number of bands expressed in the hybrid is intermediate between the number found in the two parents. Each hybrid band co-migrated with a *mercenaria* band, but only the most cathodic one co-migrated with an *adina* band.

8. *Geryonidae. Chaceon fenneri* Hc separated into six different bands, whereas *C. quinquedens* Hc separated into only five (Fig. 1B, lanes 19–20). None clearly co-migrated, and the relative amounts are distinct (Table II).

9. *Order Mysidacea.* The long-frozen bloods from the two species of *Gnathophausia* contained unusually high levels of material that was not positive for Cu, and a low concentration of Hc. Although the multiplicity of bands was clear, their resolution was so poor (Fig. 1B, lanes

7–8) that we chose not to characterize the banding patterns further. The gels are included in Fig. 1B to call attention to the distinctive behavior. The Hc bands migrated at a much slower rate than any of the other Hcs, all from decapods. Investigation of better preserved material would be of interest.

O_2 binding

Bohr plots of the data for each species are shown in Figure 2. Values for the Bohr factor, obtained as the slopes of the regression lines in Figure 2, are given in Table III, along with estimates of cooperativity.

1. *Diogenidae and Paguridae.* O_2 affinity of the four anomuran Hcs (Fig. 2; Table III, lines 1–4) differs significantly throughout most of the pH range examined, although the difference between *P. impressus* and *P. pollicarus* Hcs disappears at low pH. The Bohr factor for *Clibinarius vittatus* Hc differs considerably from those for *P. longicarpus* and *P. pollicarus* Hcs, which also differ significantly from one another (Table III, lines 1–4). Although the estimate of cooperativity for *C. vittatus* Hc is distinctive as well, the values for the three pagurids do not differ significantly from one another (Table III, lines 2–4).

2. *Geryonidae, Majidae, Grapsidae, and Mysidacea.* The Hcs of the two members of each congeneric pair of crabs had O_2 affinities that differ significantly from one another throughout the pH range examined (Fig. 2). The two mysid Hcs also differ, but only at high pH. We report these values, despite the poor condition of the material, primarily because they closely resemble the results for fresh (as well as previously frozen) material from *G. ingens* (Sanders and Childress, 1990). Only in the grapsids do values for cooperativity and pH dependence differ significantly (Table III, lines 11–12, 13–14, 39–40, and 41–42).

3. *Ocypodidae.* In *Ocypode quadrata*, O_2 affinity differed significantly from that in the *Uca* species, either above pH 7.2 (*pugilator* and *princeps monilifera*) or throughout the pH range examined (all other species). In the genus *Uca*, Hc O_2 affinities were generally distinctive but often become indistinguishable at high pH. Of particular interest (see Discussion) is a difference between *Uca pugnax* and *virens* below pH 7.4.

Bohr factors are fairly diverse (Table III, lines 15–21). If the values are arranged in series, each differs from all others except the next higher or lower one. The cooperativity figures fall into two significantly different groups: (1) the three lowest (*U. minax, pugnax,* and *virens*) and (2) the four highest (other) values.

4. *Cancridae.* Hc O_2 affinities were more alike in this than in any other genus. In 23 of the 30 possible interspecific comparisons between the six cancrid Hcs, the O_2 affinity data are indistinguishable. In no case do the values differ significantly throughout the pH range investigated. In addition, the few significant differences are small from a physiological point of view. All Bohr factors are statistically indistinguishable except those for *C. magister* and *irroratus* Hcs (Table III, lines 5–10). The cooperativity values, however, differ significantly in all comparisons except that of *C. productus* with either *magister* or *irroratus*.

5. *Portunidae.* In the following comparisons, the O_2 affinity data are statistically indistinguishable throughout the pH range examined: (1) *Callinectes similis* and *sapidus*, (2) *C. arcuatus* and *toxotes*, and (3) *C. ornatus, Ovalipes ocellatus,* and *Portunus spinnemanus*. O_2 affinity of *O. ocellatus* Hc differs from *P. gibbesii* Hc only above pH 7.4; in the remaining comparisons O_2 affinity differs significantly, either throughout the pH range or at all but the two pH extremes.

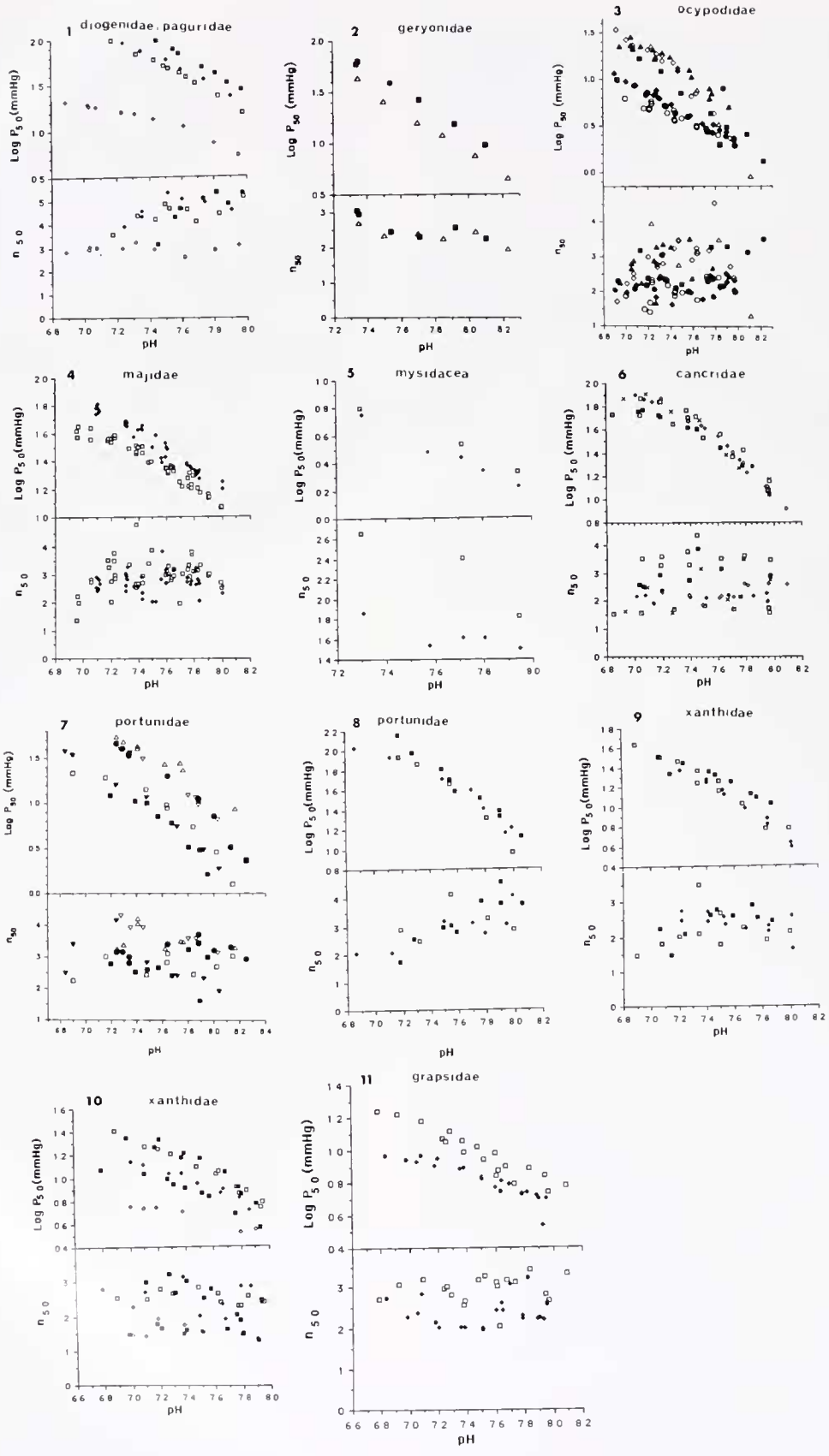
Bohr factors (Table III, lines 22–30) for the two most pH-sensitive Hcs (*C. arcuatus* and *toxotes*) differ significantly from those for the two least pH-sensitive Hcs (*P. spinnemanus* and *C. ornatus*); the Bohr factors for *C. arcuatus* and *toxotes* also differ significantly from the value for *C. sapidus*. The remaining values are not significantly different. Most of the cooperativity values are statistically homogeneous, even though *Callinectes arcuatus* and *toxotes* Hcs had been frozen and the others had not. The values for *C. ornatus* and *similis* Hcs, which do not differ from one another, are both significantly larger than the rest.

6. *Xanthidae.* The O_2 affinity values for *Cataleptodius floridanus* and *Eurypanopeus depressus* Hcs are statistically indistinguishable throughout the pH range examined, as are those for the two species of *Menippe*. *C. floridanus* Hc differed significantly from that for *M. mercenaria* except at high pH, and the *Menippe* hybrid Hc differed from the two parent species except at low pH. The difference between *Panopeus herbstii* and *obesus* Hc O_2 affinities becomes insignificant at pH 8.

Many of the Bohr factors (Table III, lines 31–38) do not differ significantly, the exceptions being (1) *P. lacustris* Hc, which is different from any other, (2) *P. herbstii* Hc, which differs from *E. depressus* and the three *Menippe* Hcs, and (3) the *Menippe* hybrid Hc, which differs from *M. adina*. The cooperativity values for *M. adina* and the *Menippe* hybrid Hcs do not differ significantly from one another. Both differ from *M. mercenaria* Hc, although the differences are small. The cooperativity value for *P. obesus* Hc is significantly greater than that for *E. depressus* Hc, again by a small margin: neither differs from *P. herbstii* Hc.

Blood PO_2 and pH

The information required to assess the physiological importance of different O_2 binding properties is available


1 diogenidae, paguridae
2 geryonidae
3 ocypodidae
4 majidae
5 mysidacea
6 cancridae
7 portunidae
8 portunidae
9 xanthidae
10 xanthidae
11 grapsidae
Log P50 (mmHg)
n50
pH

in the literature for either a single member of the taxonomic sets examined here or more than one member, but not under common environmental conditions.

In Table IV, data obtained under conditions very similar to those of the O_2 binding measurements are shown for several of the mud and fiddler crabs in our sample. In all cases the predicted oxygenation at the gill appears to be essentially complete even though experimental temperatures were fairly high. Although the predicted deoxygenation at the tissues is appreciable in most of the species, the venous reserve is high in both *Uca minax* and *U. pugnax*, perhaps because these very active runners were held in small aquaria that constrained locomotion.

Discussion

Subunit composition

The clearest findings of the survey of electrophoretic separation of crustacean Hc subunits are (1) The PAGE banding patterns are highly species specific, and (2) above the level of sibling species, the PAGE banding patterns are not especially characteristic of a taxon.

The specificity is best illustrated by following interspecific examples in which morphological differences are small, suggesting that divergence of the Hc O_2 transport system may occur quite early in speciation. As a first example, *Callinectes bellicosus* is regarded as difficult to distinguish from *C. toxotes* (Brusca, 1990). Their essentially non-overlapping but contiguous geographic ranges (Table I) raise the possibility that they are siblings; several bands in these two species co-migrated. *C. similis* is exceptionally difficult to distinguish from *C. ornatus* (Ruppert and Fox, 1988), although in this case the geographic ranges do overlap. In both cases, the differences in subunit composition are unambiguous, qualitative, and far greater than the differences between the Hc morphs found within *Callinectes sapidus* (deFur *et al.*, 1990; Mangum, 1994).

As a second example, Williams (1983) has shown that *Panopeus herbstii* (*sensu lato* Rathbun) is in fact a complex of no less than six cryptic species, which can be distinguished by careful morphological examination as well as ecological preference (Reames and Williams, 1983; Williams, 1983). Sullivan *et al.* (1983) reported that at least four of the six differ in Hc subunit composition (the other two were not examined). Our data for *P. herbstii sensu stricto* H. Milne Edwards, *obesus*, and *lacustris* strongly support this conclusion. Sullivan *et al.* (1983) briefly mentioned the existence of intraspecific polymorphism in *P. herbstii*. Although we have not yet completed our own investigation of this species, data for hundreds of individuals from the same locality and others as well

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Figure 2. O_2 binding of hemocyanins examined here. 20°C, tonometric method, unless specified otherwise.

Panel 1: Diogenidae and Paguridae. *Clibinarius vittatus* (◇), *Pagurus impressus* (⊡), *P. pollicaris* (◆), *P. longicarpus* (■). 0.05 mol l^{-1} Tris-buffered saline containing 460 mmol l^{-1} NaCl, 11 mmol l^{-1} KCl, 13 mmol l^{-1} $CaCl_2$, 20 mmol l^{-1} $MgCl_2$, 29 mmol l^{-1} $NaSO_4$ and 3 mmol l^{-1} $NaHCO_3$.

Panel 2: Geryonidae. *Chaceon fenneri* (■), *C. quinquedens* (△). 0.05 mol l^{-1} Tris-buffered saline containing 455 mmol l^{-1} NaCl, 11 mmol l^{-1} KCl, 13 mmol l^{-1} $CaCl_2$, 18 mmol l^{-1} $MgCl_2$, 22 mmol l^{-1} Na_2SO_4 and 3 mmol l^{-1} $NaHCO_3$.

Panel 3: Ocypodidae. *Uca crenulata coloradensis* (■), *U. minax* (○), *U. princeps monilifera* (△), *U. pugilator* (◇), *U. pugnax* (◆), *U. virens* (●), *Ocypode quadrata* (▲). The data for *U. crenulata coloradensis* and *princeps monilifera* were collected with the cell respiration method. 0.05 mol l^{-1} Tris-buffered saline containing 383 mmol l^{-1} NaCl, 11 mmol l^{-1} KCl, 11 mmol l^{-1} $CaCl_2$, 45 mmol l^{-1} $MgCl_2$, 40 mmol l^{-1} Na_2SO_4 and 3 mmol l^{-1} $NaHCO_3$.

Panel 4: Majidae. *Libinia dubia* (⊡), *L. emarginata* (◆). 0.05 mol l^{-1} Tris-buffered saline containing 350 mmol l^{-1} NaCl, 8 mmol l^{-1} KCl, 10 mmol l^{-1} $CaCl_2$, 42 mmol l^{-1} $MgSO_4$ and 3 mmol l^{-1} $NaHCO_3$.

Panel 5: Mycidacea. *Gnathophausia gigas* (⊡), *G. ingens* (◆). Buffered saline as in panel 2.

Panel 6: Cancridae. *Cancer antennarius* (□), *C. anthonyi* (■), *C. borealis* (⊡), *C. irroratus* (◆), *C. productus* (X). 0.05 mol l^{-1} Tris-buffered saline containing 441 mmol l^{-1} NaCl, 15 mmol l^{-1} KCl, 11 mmol l^{-1} $CaCl_2$, 40 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} Na_2SO_4 and 3 mmol l^{-1} $NaHCO_3$.

Panel 7: Portunidae. *Callinectes arcuatus* (□), *C. bellicosus* (■), *C. ornatus* (▽), *C. sapidus* (●), *C. similis* (△), *C. toxotes* (▼). Data collected at 25°C with the cell respiration method. Buffered saline as in panel 2.

Panel 8: Portunidae. *Ovalipes ocellatus* (⊡), *Portunus spinnemanus* (◆), *P. gibbesii* (■). Buffered saline as in panel 2.

Panel 9: Xanthidae. *Menippe adina* (◆), *M. mercenaria* (⊡), *M. adina–mercenaria* hybrid (■). 0.05 mol l^{-1} Tris-buffered saline containing 350 mmol l^{-1} NaCl, 7 mmol l^{-1} KCl, 11 mmol l^{-1} $CaCl_2$, 33 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} Na_2SO_4 and 3 mmol l^{-1} $NaHCO_3$.

Panel 10: Xanthidae. *Cataleptodius floridanus* (■), *Eurypanopeus depressus* (⊡), *Panopeus herbstii* (◆), *P. lacustris* (◇), *P. obesus* (◘). Buffered saline as in panel 9.

Panel 11: Grapsidae. *Sesarma cinereum* (⊡), *S. reticulatum* (◆). 0.05 mol l^{-1} Tris-buffered saline containing 383 mol l^{-1} NaCl, 11 mmol l^{-1} KCl, 16 mmol l^{-1} $CaCl_2$, 45 mmol l^{-1} $MgCl_2$, 40 mmol l^{-1} Na_2SO_4 and 3 mmol l^{-1} $NaHCO_3$.

Table III

Bohr factors and cooperativity values

Species	Number of individuals (n)	Bohr Factor $\Delta\log p_{50}/\Delta pH$ (±95% CI)	Cooperativity n_{50} ($\bar{x} \pm SE$)
Order Decapoda			
Infraorder Anomura			
Family Diogenidae			
1. *Clibinarius vittatus*	7	−0.54 ± 0.10	2.96 ± 0.02
Family Paguridae			
2. *Pagurus impressus*	6	−0.98 ± 0.07	4.51 ± 0.04
3. *Pagurus longicarpus*	47	−1.06 ± 0.06	4.71 ± 0.11
4. *Pagurus pollicaris*	5	−0.88 ± 0.10	4.71 ± 0.07
Infraorder Brachyura			
Family Cancridae			
5. *Cancer antennarius*	12	−0.76 ± 0.17	3.61 ± 0.04
6. *Cancer anthonyii*	12	−0.74 ± 0.16	2.95 ± 0.05
7. *Cancer borealis*	6	−0.61 ± 0.11	2.08 ± 0.02
8. *Cancer irroratus*	6	−0.83 ± 0.13	2.12 ± 0.01
9. *Cancer magister*	4	−1.05 ± 0.11	2.36 ± 0.03
10. *Cancer productus*	1	−0.77 ± 0.24	2.25 ± 0.08
Family Grapsidae			
11. *Sesarma cinereum*	42	−0.43 ± 0.07	3.17 ± 0.07
12. *Sesarma reticulatum*	22	−0.30 ± 0.06	2.28 ± 0.07
Family Majidae			
13. *Libinia dubia*	24	−0.60 ± 0.04	2.85 ± 0.07
14. *Libinia emarginata*	15	−0.64 ± 0.04	2.70 ± 0.06
Family Ocypodidae			
15. *Ocypode quadrata*	9	−0.83 ± 0.12	2.66 ± 0.12
16. *Uca crenulata coloradensis*	7	−0.97 ± 0.13	3.08 ± 0.16
17. *Uca minax*	11	−0.50 ± 0.09	2.18 ± 0.06
18. *Uca princeps monilifera*	5	−1.60 ± 0.35	2.93 ± 0.25
19. *Uca pugilator*	102	−1.17 ± 0.16	2.82 ± 0.13
20. *Uca pugnax*	65	−0.63 ± 0.06	2.20 ± 0.18
21. *Uca virens*	24	−0.70 ± 0.05	2.18 ± 0.05
Family Portunidae			
22. *Ovalipes ocellatus*	4	−1.19 ± 0.30	3.14 ± 0.12
23. *Portunus gibbesii*	6	−1.09 ± 0.14	3.23 ± 0.09
24. *Portunus spinimanus*	2	−0.77 ± 0.29	2.91 ± 0.12
25. *Callinectes arcuatus**	12	−1.51 ± 0.34	2.73 ± 0.11
26. *Callinectes bellicosus**	10	−0.97 ± 0.34	2.45 ± 0.10
27. *Callinectes ornatus**	10	−0.82 ± 0.16	3.68 ± 0.22
28. *Callinectes sapidus**	6	−0.98 ± 0.10	3.18 ± 0.08
29. *Callinectes similis**	5	−1.03 ± 0.18	3.54 ± 0.20
30. *Callinectes toxotes**	12	−1.38 ± 0.14	2.64 ± 0.27
Family Xanthidae			
31. *Menippe adina*	6	−0.96 ± 0.11	2.39 ± 0.03
32. *Menippe mercenaria*	2	−0.85 ± 0.14	2.17 ± 0.06
33. Hybrid	2	−0.63 ± 0.09	2.41 ± 0.05
34. *Panopeus herbstii*	30	−0.45 ± 0.14	2.61 ± 0.05
35. *Panopeus obesus*	12	−0.45 ± 0.14	2.74 ± 0.04
36. *Panopeus lacustris*	7	−0.32 ± 0.07	
37. *Eurypanopeus depressus*	29	−0.61 ± 0.07	2.55 ± 0.01
38. *Cataleptodius floridanus*	18	−0.63 ± 0.15	
Family Geryonidae			
39. *Chaceon fenneri*	10	−1.06 ± 0.07	2.64 ± 0.12
40. *Chaceon quinquedens*	10	−1.08 ± 0.10	2.35 ± 0.10
Order Mysidacea			
41. *Gnathophausia gigas*	3	−0.72 ± 0.15	
42. *Gnathophausia ingens*	2	−0.80 ± 0.15	

* 25°C, cell respiration method.

(K. T. White and C. P. Mangum, unpub. obs.) confirm this inference. Once again, none of the morphs could be confused with a pattern reported here for another species in the Xanthidae.

Additional evidence in support of the specificity of PAGE banding of Hc monomers has been obtained for sibling species of the lobster genus *Homarus* and their hybrids (Mangum, 1993b) and for several other sibling species, including stone crabs of the genus *Menippe* investigated here (C. P. Mangum, unpub. data).

Of the sibling species in the present sample, the difference between only one pair remains tentative. The status of *Uca virens* (Salmon and Astiades) remains unresolved. It is variously regarded as a synonym of *U. rapax*, a subspecies of *U. pugnax*, or a separate and valid species (reviewed by Barnwell and Thurman, 1984; Salmon and Kettler, 1987). The most cathodic band in the present material from *U. virens* and *pugnax* differs greatly in quantity and slightly in migration rate. Although more recent findings make it clear that the morphs found within *Uca pugnax* do not include a phenotype that could be confused with those found in a sample of *U. rapax/virens* from the northern Gulf of Mexico (C. P. Mangum, unpub. data), we have not yet compared *Uca rapax* and *U. virens* in depth.

At higher taxonomic levels, only a few general features are common to a set. One is the considerable monomeric heterogeneity found in the portunid genera *Callinectes* and *Portunus*. Another is the sorting of bands into three electrophoretic categories with characteristic migration rates in the genus *Cancer*, as originally reported by Markl (1986).

Native PAGE banding patterns, however, are not especially characteristic of a genus, much less a family. In the six species of *Cancer*, electrophoretic patterns differ as much from one another as do those in different families. In this example, the dissimilarities may entail the distance of the interspecific relationships. One would expect the patterns to be most similar in recently separated species with wholly allopatric geographic ranges, such as *Homarus americanus* and *gammarus* (Mangum, 1993b). In contrast, the cancrids in the present sample are either separated by a continent or they are sympatric, suggesting far more phylogenetic distance between species. Similarly, the sympatric species of *Sesarma* and *Libinia*, in each of which the Hc phenotype is quite different from that of its congener, are not very closely related to one another (D. L. Felder, pers. comm.). In distantly related species, such as *Portunus gibbesii* and *Callinectes ornatus*, co-migratory bands may well prove to represent unlike polypeptides that happen to be similar in net charge.

Respiratory properties

The clearest findings to emerge from the survey of the O_2-binding properties examined are that within a taxo-

Table IV

In vivo *respiratory variables (23°C)*

Species	Medium	PaO_2 (Torr)	PvO_2	PaH	PvH	Hc_aO_2 (%)	Hc_vO_2
Family Ocypodidae							
Uca minax	water	88 ± 9 (4)	17 ± 1 (4)	7.55 ± 0.05 (5)	7.55 ± 0.03 (7)	100	88
Uca pugilator	(24 h)	63 ± 4 (7)	20 (2)	7.48 ± 0.03 (16)	7.40 ± 0.05 (10)	99	63
Uca pugnax		95 ± 10 (6)	10 ± 3 (4)	7.53 ± 0.07 (8)	7.43 ± 0.08 (8)	100	73
Uca minax	air	63 ± 6 (64)	12 ± 1 (5)	7.43 ± 0.08 (4)	7.35 ± 0.04 (8)	100	73
Uca pugilator	(24 h)	72 ± 13 (4)	16 ± 2 (4)	7.42 ± 0.02 (4)	7.39 ± 0.03 (10)	100	49
Uca pugnax		72 ± 5 (6)	10 ± 2 (5)	7.56 ± 0.05 (6)	7.45 ± 0.04 (11)	100	73
Family Xanthidae							
Eurypanopeus depressus	water	101 ± 8 (6)	16 ± 2 (3)	7.44 ± 0.05 (6)	7.27 ± 0.20 (5)	100	49
Panopeus herbstii		77 ± 6 (14)	18 ± 2 (10)		7.38*	99	61
Panopeus obesus		135 (2)	9 ± 2 (3)	7.55 ± 0.08 (2)	7.56 ± 0.05 (4)	100	75

a = postbranchial, v = prebranchial. Mean ± SE (*n*)
* From Mangum (1973).

nomic set, (1) O_2 affinity varies the most in closely related species; (2) cooperativity also varies but not as often; and (3) the pH dependence of O_2 affinity is the most highly conserved.

Although most of the Hcs in the present sample have O_2 affinities that are species specific, there are numerous exceptions. The portunid Hcs, which are always composed of a large number of different subunits, can have very different O_2 affinities, but O_2 affinities can also be identical. Moreover, the cancrids, which have quite diverse subunit patterns, have the least different O_2 affinities.

The comparison of sibling and cryptic species is also of interest here. The difference in O_2-binding properties of Hcs from the cryptors *Panopeus herbstii* and *P. obesus* suggests divergence at the physiological level. These differences are also adaptive in terms of the magnitude of O_2 transport. *P. herbstii,* with its higher prebranchial blood PO_2, has the lower Hc O_2 affinity; *P. obesus,* with its lower prebranchial blood PO_2, has the higher Hc O_2 affinity. The result is that deoxygenation at the tissues differs less than it would if O_2 affinity were the same.

Cooperativity is distinctive in only one taxonomic set, the pagurids, in which it is unusually high. Otherwise, the values are typical of the crustacean Hcs. Many of the differences within a taxonomic set, though significant, are small. Within the Cancridae, however, the range is fairly large. It would be interesting to learn whether this diversity is related to the notable heterogeneity and interspecific diversity of subunit composition.

Bohr factors are the most highly conserved of the respiratory properties examined here. In general, they appear to be at least somewhat characteristic of a taxonomic set. For example, in the two grapsids the pH sensitivity of O_2 affinity is unusually small for crustaceans, and in the two majids it is only moderate. The greater pH dependence of the portunid, cancrid, and pagurid Hcs is typical of crustacean Hcs. But this generalization is not entirely reliable, as indicated by the diversity within the *Panopeus* species complex.

Relationship between subunit composition and respiratory properties

Finally, the survey of 44 crustacean Hcs failed to reveal a simple relationship between intrinsic respiratory properties and either qualitative or quantitative aspects of electrophoretic subunit composition. Instead, it suggested that respiratory properties are strongly selected by factors of immediate relevance to the particular species, and that the response is constrained very little, if at all, by monomeric subunit composition.

For example, in *Callinectes* two pairs of congeneric Hcs have identical O_2-binding properties but very different subunit compositions. In *C. sapidus* and *similis*—sympatric species that are easily distinguished morphologically—O_2 binding properties are indistinguishable, and yet only one of six bands co-migrated. Moreover, the diagnostic bands in *C. sapidus* included nos. 3 and 6, which are known to influence O_2 affinity (Mangum and Rainer, 1988; deFur *et al.,* 1990; Mangum *et al.,* 1991). The differences in oxygenation properties of *Uca minax, U. pugnax,* and *U. virens* Hcs are also small, in spite of quite different subunit compositions. At more distant levels of taxonomic separation, the Hcs of the xanthids *Eurypanopeus depressus* and *Cataleptodius floridanus* were as different as any in subunit composition but as similar as any in O_2 binding. On the other hand, different subunit compositions may be responsible for functional differences between sibling species such as the stone crabs investigated here and the lobsters examined earlier (Mangum, 1993b).

In general, O_2 affinity appeared to be more closely related to thermal properties of the environment than to subunit composition. The family Cancridae, for example, with its similar O_2 affinities and dissimilar subunit patterns, is essentially a boreal to temperate zone family, with its species found in offshore, colder waters of the latter; O_2 affinity was uniformly low. In contrast, *Callinectes,* with its widely differing O_2 affinities, is believed to be a genus that is of tropical origin and is rapidly speciating northwards; O_2 affinity was lower in the more northern and higher in the more southern species. The decreasing O_2 affinity with increasing latitude in some of the species of *Panopeus* and *Uca* also supports this hypothesis. Although the low O_2 affinities in the southeastern Atlantic coast species of *Chaceon* might appear to be exceptional, in fact both inhabit offshore, cold waters. In addition, the species with the lower Hc O_2 affinity is found at the greater depths and thus at the lower temperature. In these examples, O_2 affinities differ less at mean environmental temperatures than they would if P_{50} values were identical (see also Mangum, 1982; Mauro and Mangum, 1982). Moreover, the existence of very similar respiratory properties in quite distantly related species indicates selection for common functional properties, which can be brought about by very different Hc monomers.

We do not intend to suggest that environmental temperature is the sole selection pressure on intrinsic O_2 affinity, or even that the environment is the sole determinant. A thermal interpretation is not consistent with the geographic ranges of the two species of grapsids or the three temperate zone species of *Uca.* According to Fotheringham and Brunenmeister (1975), the grapsid *Sesarma reticulatum,* which had the higher Hc O_2 affinity, carries on gas exchange in the (perhaps hypoxic) water filling a deep burrow, whereas *S. cinereum,* with its lower O_2 affinity, is basically an air-breather with a larger gill surface area than its congener. The difference between *Uca pugilator* and *U. pugnax,* however, is fairly large despite similar branchial surface areas (Pearse, 1950) and geographic ranges. In this case burrow PO_2 may differentiate the sand (*U. pugilator*) and the mud (*U. pugnax*) fiddler crabs.

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